IDENTIFICATION OF THE LINKAGE BETWEEN G PROTEINS AND ERYTHROCYTE PROTEIN BAND 3

F.A. Carvalho, J. Martins e Silva, C. Saldanha*

Instituto de Biopatologia Química, Unidade de Biopatologia Vascular – Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, 1649-028 Lisboa, Portugal
* filomenacarvalho@fm.ul.pt

ABSTRACT

Circulating acetylcholine (natural substrate of acetylcholinesterase) is able to modulate the microcirculatory blood flow by controlling nitric oxide (NO) intracellular mobilization, its metabolism (NO_x) and its release from erythrocytes. In reverse, velnacrine maleate plays a competitive role as an acetylcholinesterase (AChE) inhibitor decreasing NO-mediated erythrocyte responses.

In our previous studies we hypothesis that a possible response's mechanism lies on the NO translocation among nitrosylated molecules through a protein G linked to band 3 protein. Band 3 phosphorylated/dephosphorylated states are processed by major tyrosine-kinases (PTK) and phosphotyrosine-phosphatases (PTP).

So we intend to identify the G protein form that could be linked to the protein band 3 and to know each protein G sub-units (α,β,γ) are related to the activation or inhibition of acetylcholinesterase complex form and phosphorylation band 3 degree states. For this propose we made Western blotting analysis using primary antibodies to different protein G sub-units such as anti-protein G_β, anti-protein $G\alpha_{i1/2}$, anti-protein $G\alpha_{i3}$, anti-protein $G\alpha_{i3}/G\alpha_0$, anti protein $G\alpha_S$ and anti-protein $G\alpha_{q/11}$. We could identify on erythrocytes membrane soluble extracts possible linkage between protein $G\alpha_{i1/2}$ and/or protein G_β with protein band 3. The results were then confirmed by immunoprecipitation of this two protein G sub-units with following analysis by Western blot using antibodies against protein band 3 (C-terminal) and band 3 (N-terminal).

From all the blood samples aliquots studied we could concluded that G protein sub-units $G\alpha_{i1/2}$ and G_β could be linked with band 3 C-terminal site and only $G\alpha_{i1/2}$ are bonded with band 3 N-terminal. The connection between sub-unit G_β and band 3 at C-terminal was not seemed. Moreover when erythrocyte acetylcholinesterase was stimulated with acetylcholine and when is present with PTK inhibitors there was an increase of the expression of the linkage between $G\alpha_{i1/2}$ – Band 3 (C- and N-

terminal) and G_{β} - Band 3 (C –terminal). These two conformational states of G protein sub-units seem to be related with the phosphorylation band 3 protein states.

Heterotrimeric G proteins mediate signal transduction pathways; moreover it is known that stimulation of G_i results in inhibition of adenylyl cyclase and ATP release from these cells. So human erythrocyte $NO_{(x)}$ mobilization levels may occur under the influence of AChE effectors by mechanisms related to the degree of band 3-phosphorylation and activation of adenylyl cyclase. These events underlying NO translocation/mobilization changes may occur on microcirculation disease, a target upon which novel coadjuvant drugs may become accessible.

INTRODUCTION

We recently hypothesize the involvement of AChE effectors enzymatic complexes in a band 3-dependent signal transduction pathway related to changes in the intracellular NO metabolism and the production of its chief metabolites, nitrites and nitrates[1]. Our hypothesis is based on the acetylcholinesterase role on signal transduction mechanism in response to action of ACh or VM on NO production (and its metabolites) in human erythrocytes suspensions that could be

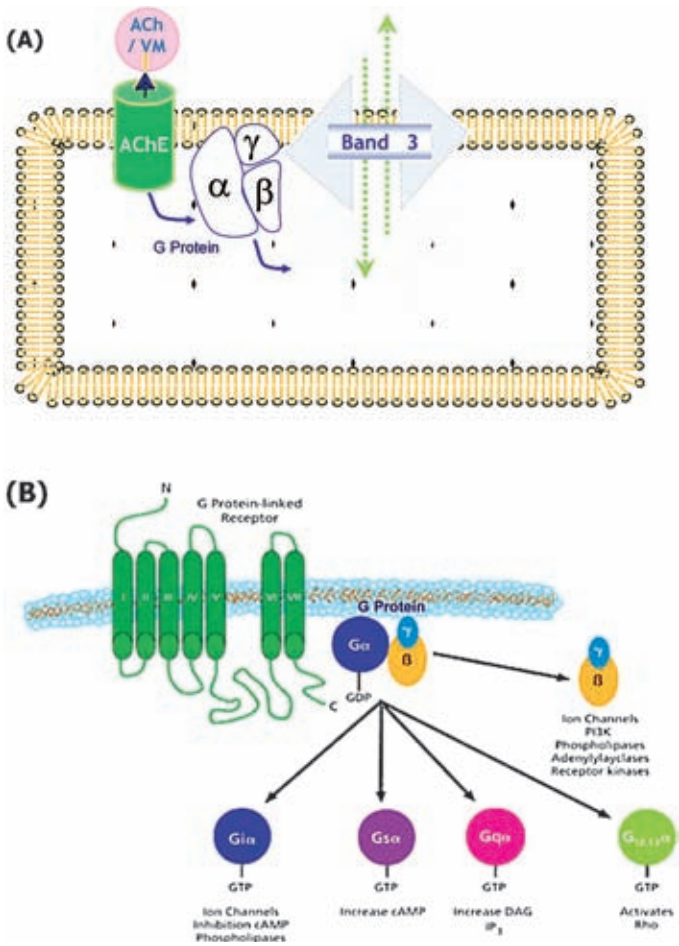
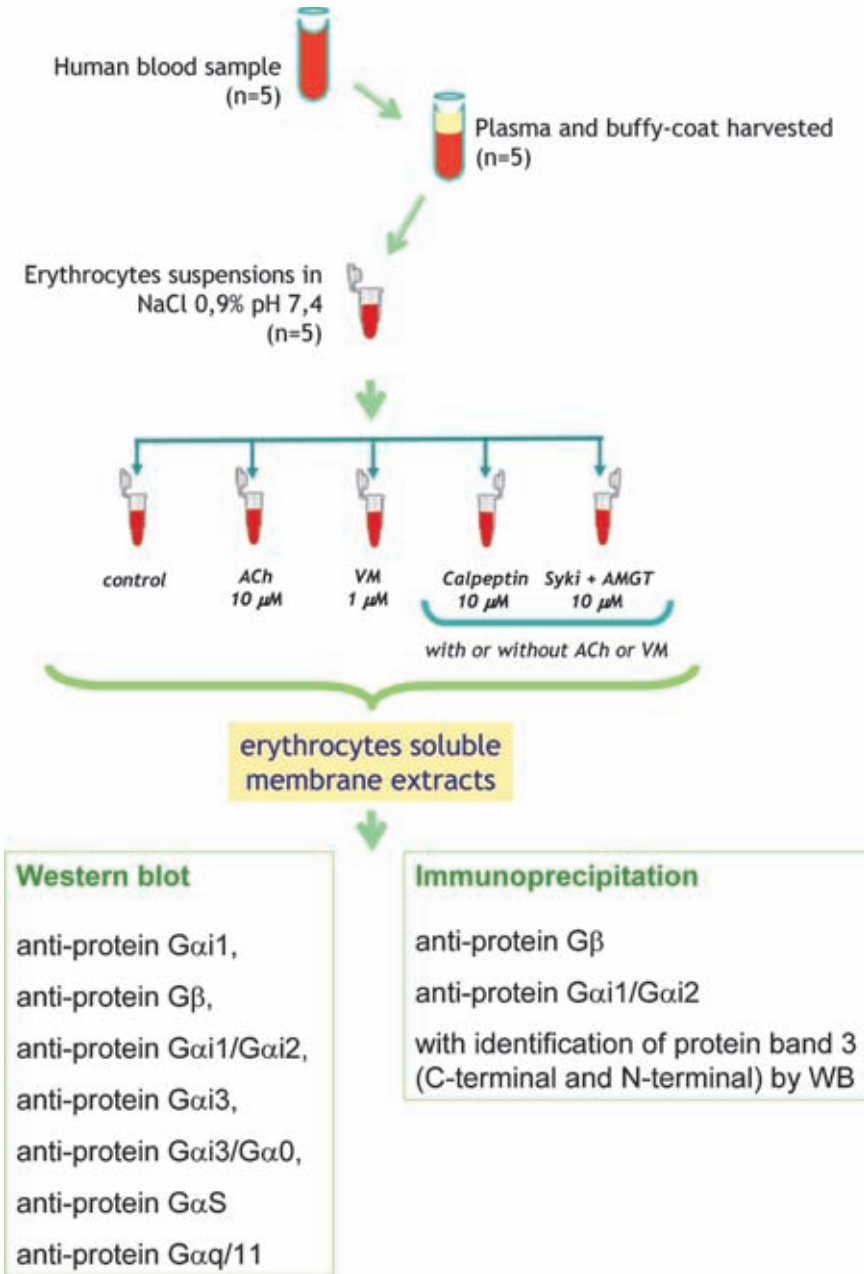


Fig. 1 – **(A)** Presentation of the hypothesis for acetylcholinesterase role on signal transduction mechanism in response to action of ACh or VM on NO production (and its metabolites) in human erythrocytes suspensions is mediated by transnitrosilation processes between phosphorylated / dephosphorylated band 3 when PTK or PTP enzymatic activity are inhibited. This process could be dependent of a G protein (α,β,γ) (Carvalho F.A. et al (2004).
(B) Receptors coupled to heterotrimeric GTP-binding proteins (G proteins) are integral transmembrane proteins that transduce extracellular signals to the cell interior. Receptor occupation promotes interaction between the receptor and the G protein on the interior surface of the membrane. This induces an exchange of GDP for GTP on the G protein a subunit and dissociation of the a subunit from the bg heterodimer. Depending on its isoform, the GTP-a subunit complex mediates intracellular signaling either indirectly by acting on effector molecules such as adenylyl cyclase (AC) or phospholipase C (PLC), or directly by regulating ion channel or kinase function.[2]

related to the transnitrosilation process between phosphorylated / dephosphorylated band 3 and SNOHb could be associated to an unidentified mechanism mediated by formation of AChE-ACh (more active) or AChE-VM (less active) complexes, when PTK or PTP enzymatic activity is inhibited. When we proposed this signal transduction mechanism we thought that this process could also be dependent of a G protein. So the aim of this study was to identify the G protein form that could be linked to the protein band 3 and to know what protein G sub-units (α,β,γ) are related to the activation or inhibition of acetylcholinesterase and band 3 phosphorylation degree states.

EXPERIMENTAL DESIGN



RESULTS

We could observe from Western blotting analysis (vd. Fig. 2) that protein Gβ and protein Gαi1/Gαi2 could be linked to the erythrocyte protein band 3. All the others protein G isoforms tested are identified on erythrocytes membrane extracts but there is no evidence that could be linked to protein band 3. The immunoprecipitation analysis between the protein Gβ or protein Gαi1/Gαi2 and protein band 3 confirm the previous Western blotting results (vd. Fig. 3 and 4)

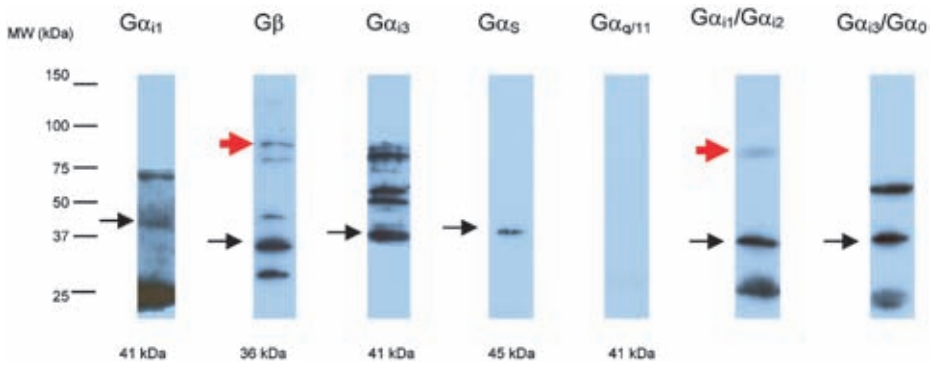


Fig. 2 – Detection of protein Gαi1, protein Gβ, protein Gαi1/Gαi2, protein Gαi3, protein Gαi3/Gα0, protein GαS and protein Gαq/11 on erythrocyte membrane soluble extracts (control) by immunoblotting.

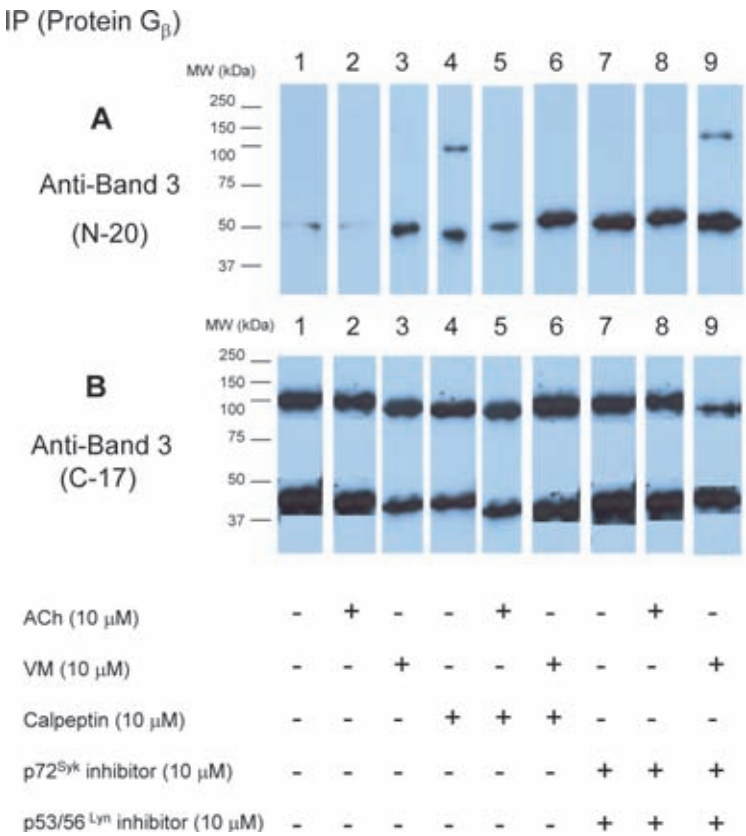


Fig. 3 – Immunoprecipitation of protein Gβ on erythrocyte membrane soluble extracts previously incubated with control (lane 1), ACh 10 μM (lane 2), VM 10 μM (lane 3), p72syk inhibitor 10 μM (lane 4) with ACh (lane 5) or VM (lane 6) and p53/56lyn inhibitor 10 μM (lane 7) with ACh (lane 8) or VM (lane 9). The immunoprecipitate was electrophoresed and immunoblotted with anti-band 3 antibodies for C-terminal and N-terminal.

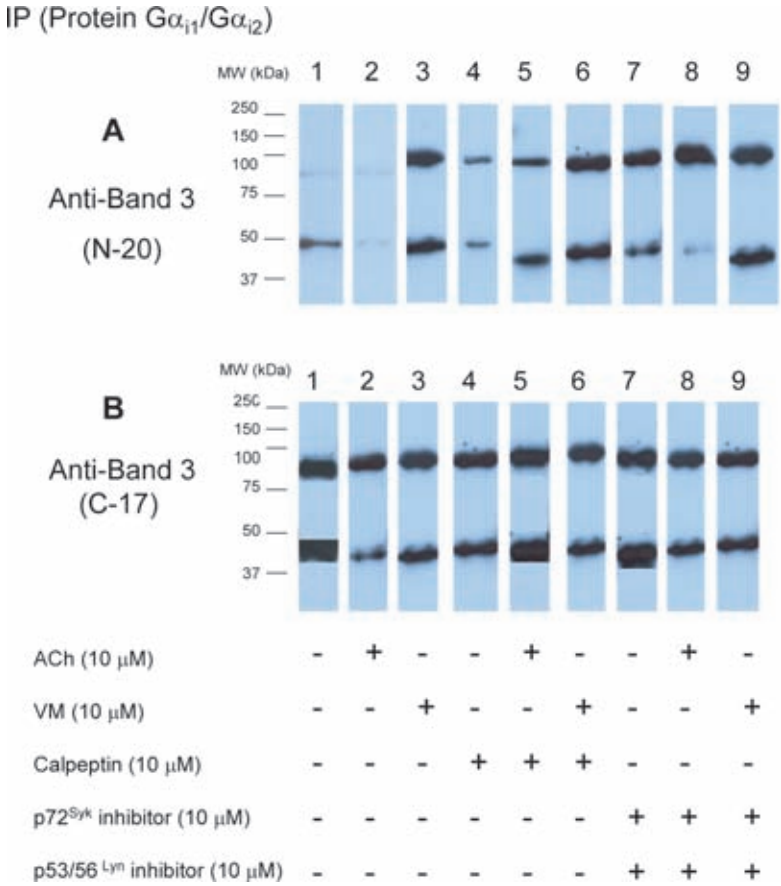


Fig. 4 – Immunoprecipitation of protein Gαi1/Gαi2 on erythrocyte membrane soluble extracts previously incubated with control (lane 1), ACh 10 μM (lane 2), VM 10 μM (lane 3), p72syk inhibitor 10 μM (lane 4) with ACh (lane 5) or VM (lane 6) and p53/56lyn inhibitor 10 μM (lane 7) with ACh (lane 8) or VM (lane 9). The immunoprecipitate was after submitted to Western blotting with anti-band 3 antibodies for C-terminal and N-terminal.

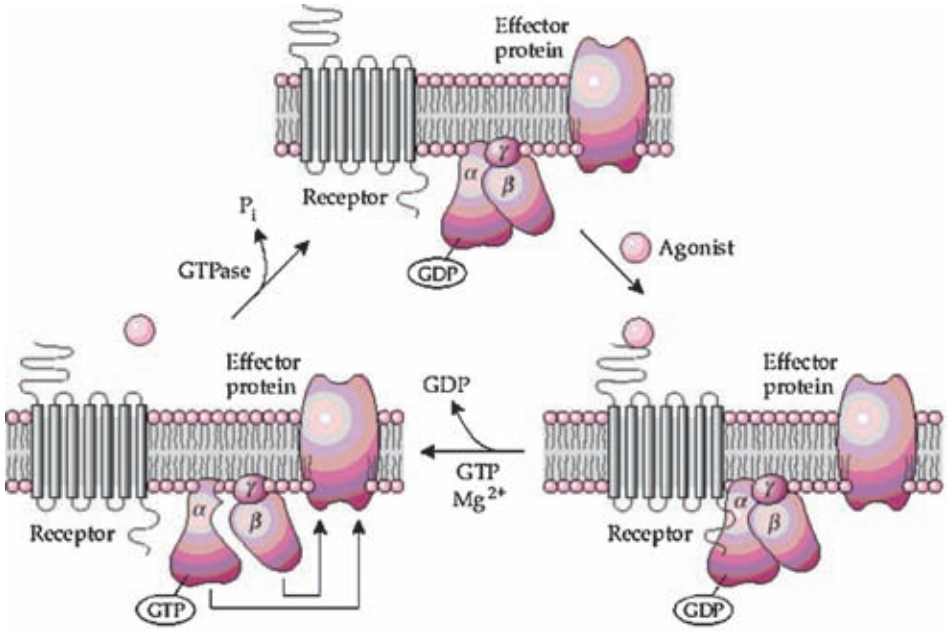
CONCLUSIONS

The results of this work allow us to

- A possible interaction between protein Gαi1/Gαi2 and/or protein Gβ and protein band 3 on erythrocytes membrane
- Band 3 C-terminal: both protein Gαi1/Gαi2 and protein Gβ may be bonded for immunoprecipitation
- Band 3 N-terminal: protein Gαi1/Gαi2 may be bonded for immunoprecipitation; Gβ may be bonded only when band 3 is phosphorylated and when PTK inhibitors are incubated with velnacrine
- Stimulation with ACh (with or without PTK inhibitors) – increase of the linkage Gαi1/Gαi2 – band 3 (C-terminal) expression
- The observed two different conformational G protein sub-units stages seems to be related with the phosphorylation band 3 protein

From these results we proposed that a possible G protein sub-unit conformational mechanism that could be related with our findings could be the one indicated here.[3]

The heterotrimeric G protein Gi, participates in the ATP release from erythrocytes[4]. The expression of G_{i2} is reduced in the erythrocyte membranes of humans with type 2 diabetes[5]. It was also verified a decreased of G proteins (Gαi, Gαo and Gβ) in hypertensive subjects[6]. All together these results suggests that this defect in erythrocyte physiology could lead to a reduced stimulus for endogenous NO synthesis in the microvasculature,



In summary, our results allows us to confirm that G_i is a necessary component of the signal transduction pathway that seems to be linked to the protein band 3 phosphorylation degree states, and related to the activation or inhibition of acetylcholinesterase complex and supports our proposed erythrocyte NO translocation mechanism.

REFERENCES

1. Carvalho FA, Mesquita R, Martins-Silva J, Saldanha C. Acetylcholine and choline effects on erythrocyte nitrite and nitrate levels. J Appl Toxicol 2004, 24(6):419–427.
2. www.sigmaaldrich.com/img/assets/6460/G_protein.gif
3. www.chemistry.emory.edu/.../images/fig_6.23.gif
4. Olearczyk JJ, Stephenson AH, Lonigro AJ, Sprague RS. Heterotrimeric G protein Gi is involved in a signal transduction pathway for ATP release from erythrocytes. Am J Physiol 2004, 286:H940–H945
5. Sprague RS, Stephenson AH, Bowles EA, Stumpf MS, Lonigro AJ. Reduced expression of G(i) in erythrocytes of humans with type 2 diabetes is associated with impairment of both cAMP generation and ATP release. Diabetes 2006; 55:3588-93.
6. Escribá PV, Sánchez-Dominguez JM, Alemany R, Perona JS, Ruiz-Gutiérrez V. Alteration of lipids, G proteins, and PKC in cell membranes of elderly hypertensives. Hypertension 2003, 41:176-82.